



U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

USAMRICD-TR-03-01

Clinical Drug Treatment of Edemagenic Gas-induced Lung Injury

Alfred M. Sciuto
Holcombe H. Hurt

February 2003

20031103 028

Approved for public release; distribution unlimited

U.S. Army Medical Research
Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5400

DISPOSITION INSTRUCTIONS:

Destroy this report when no longer needed. Do not return to the originator.

DISCLAIMERS:

The opinions or assertions contained herein are the private views of the author(s) and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulations mandated for an AAALAC accredited facility.

The use of trade names does not constitute an official endorsement or approval of the use of such commercial hardware or software. This document may not be cited for purposes of advertisement.

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 2003		2. REPORT TYPE technical		3. DATES COVERED (From - To) 1991-1996	
4. TITLE AND SUBTITLE Clinical Drug Treatment of Edemagenic Gas-induced Lung Injury				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER 61384	
6. AUTHOR(S) Sciuto, AM, Hurt HH				5d. PROJECT NUMBER	
				5e. TASK NUMBER TC1	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)				8. PERFORMING ORGANIZATION REPORT NUMBER USAMRICD-TR-03-01	
US Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-PN 3100 Ricketts Point Road		Aberdeen Proving Ground, MD 21010-5400			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Institute of Chemical Defense ATTN: MCMR-UV-RC 3100 Ricketts Point Road				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Studies were performed to address treatment against phosgene gas. Efficacy was judged by examining post-treatment (PTx) effects on pulmonary edema (PE) i.e. lung weight gain (LWG), survival rates (SR), odds ratios (OR), and glutathione (GSH) redox state, and lipid peroxidation (LP). N-acetylcysteine (NAC), ibuprofen (IBU), aminophylline (AMIN), and isoproterenol (ISO) were studied in the isolated perfused rabbit lung model (IPRLM) and mouse model. Intratracheal (IT) NAC delivered 1 h after phosgene exposure lowered pulmonary artery pressure (Ppa), LWG, leukotrienes (LTs), LP, and oxidized GSH. Using the IPRLM, AMIN after phosgene exposure significantly reduced LP and LTs, and LWG. Ptx with ISO in the IPRLM by combined intravascular (IV) or intratracheal (IT) route 1 h after phosgene lowered Ppa, tracheal pressure, LWG, and LTs. In mice IBU was administered i.p. 20 min after phosgene at 0, 3, 9, or 15 mg/mouse. At 5 h, a second IBU injection was given. The OR was 5 for the 9/4.5 IBU group at 12 h and 13 for the 15/7.5 mg IBU. IBU enhanced mouse SR by reducing PE, LP, and GSH depletion. Treatment of phosgene injury involves early intervention that reduces LP, maintains GSH, and prevents the release of LTs responsible for PE.					
15. SUBJECT TERMS phosgene, lung, edema, lipid peroxidation, leukotrienes, animals, isoproterenol, n-acetylcysteine, aminophylline, ibuprofen, survival rates, odds ratio, glutathione					
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Alfred M. Sciuto	
a. REPORT UNCLASSIFIED	b. ABSTRACT UNCLASSIFIED	c. THIS PAGE UNCLASSIFIED	UNLIMITED	30	19b. TELEPHONE NUMBER (include area code) 410-436-5115

Abstract

A series of studies was performed to address treatment against the former chemical warfare edemagenic gas phosgene. Both *in situ* and *in vivo* models were used to assess the efficacy of post-exposure treatment of phosgene-induced lung injury using clinically existing drugs. The degree of efficacy was judged by examining treatment effects on pulmonary edema formation (PEF) as measured by wet/dry weight (WW/DW) ratios, real time (*in situ*) lung weight gain (LWG), survival rates (SR), odds ratios, and glutathione (GSH) redox states. Drugs included N-acetylcysteine (NAC), ibuprofen (IBU), aminophylline (AMIN), and isoproterenol (ISO). Using the *in situ* isolated perfused rabbit lung model (IPRLM), intratracheal (IT) NAC (40 mg/kg bolus) delivered 45-60 min after phosgene exposure (650 mg/m³) for 10 min lowered pulmonary artery pressure, LWG, leukotrienes LTC₄/D₄/E₄, lipid peroxidation, and oxidized GSH. We concluded that NAC protected against phosgene-induced lung injury by acting as an antioxidant by maintaining protective levels of GSH, reducing both lipid peroxidation and production of arachidonic acid metabolites. Also in IPRLM, administration of AMIN (30 mg/kg) 80-90 min after phosgene exposure significantly reduced lipid peroxidation and perfusate LTC₄/D₄/E₄, LWG and prevented phosgene-induced decreases in lung tissue cAMP. These data suggest that protective mechanisms observed with AMIN involve decreased LTC₄/D₄/E₄-mediated pulmonary capillary permeability and attenuated lipid peroxidation. Direct anti-permeability effects of AMIN-induced up-regulation of cAMP on cellular contraction may also be important in protection against phosgene-induced lung injury. Post-treatment with ISO in the IPRLM by either combined intravascular (IV- infused into pulmonary artery @ 24 µg/min infused)+IT (24 µg bolus) or IT route alone 50-60 min after phosgene exposure significantly lowered pulmonary artery pressure, tracheal pressure, and LWG. ISO treatment significantly enhanced GSH products or maintained protective levels when compared with results from phosgene-exposed only rabbits. These data suggest that protective mechanisms for ISO involve reduction in vascular pressure, decreased LTC₄/D₄/E₄-mediated pulmonary capillary permeability, and favorably maintained lung tissue GSH redox states. In *in vivo* male mouse (CD-1, 25-30 g) studies IBU was administered i.p. within 20 min after a lethal dose of phosgene (32 mg/m³ for 20 min) at 0 (saline), 3, 9, or 15 mg/mouse. Five h later, a second IBU injection was given but at half the original doses (0, 1.5, 4.5, and 7.5 mg/mouse); therefore, these treatment groups are now referred to as the 0/0, 3/1.5, 9/4.5 and 15/7.5 mg IBU/mouse groups. SRs and odds ratios were calculated for each dose at 12 and 24 h. The 12-h survival was 63% for 9/4.5 mg IBU and 82% for the 15/7.5 mg IBU groups compared with 25% for saline-treated phosgene-exposed mice. At 24 h, those survival rates were reduced to 19%, 19% and 6%, respectively. In the 15/7.5 mg IBU group, lung WW/DW ratios were significantly lower than in saline-treated mice at 12 h. Lipid peroxidation was lower only for the 9/4.5 mg IBU dose; however, non-protein sulphydryls (a measure of GSH) were greater across all IBU doses. The odds ratio was five for the 9/4.5 IBU group at 12 h and 13 for the 15/7.5 mg IBU group compared with 3.5 for both groups at 24 h. IBU post-treatment increased the survival of mice at 12 h by reducing PEF, lipid peroxidation, and GSH depletion. In conclusion, effective treatment of phosgene-induced lung injury involves early post-exposure intervention that could reduce free radical species responsible for lipid peroxidation, correct the imbalance in the GSH redox state, and prevent the release of biological mediators such as leukotrienes, which are accountable for increased permeability.

Introduction

Phosgene (COCl_2), an irritant and choking gas, was the primary weaponized respiratory threat agent in use during World War I. Phosgene was used alone, but it was also mixed with other gases such as chlorine. As such, phosgene gassing during WW I caused men to lose 311,000 days due to hospitalization, an astonishing 852 man-years (1).

While phosgene has been a potential military and terrorist threat for many years because it is easily and cheaply produced, it is also an occupational and an environmental hazard. Since WWII phosgene has gained widespread use in industry as a chemical precursor in the production of pharmaceuticals, dyes, pesticides, and polyurethane for foam rubber products. Data compiled through 1990 indicate that nearly one million tons of phosgene were produced in the US every year (2). Although phosgene is nearly completely consumed during industrial use, it can be released through failed processes. Phosgene is an environmental pollutant and has been detected in ambient air samples (3). Phosgene can be formed by the thermal decomposition of chlorinated hydrocarbons and poses a threat for welders, refrigeration mechanics, and car repairmen (4). Commonly used industrial degreasers contain chlorinated hydrocarbons, such as perchloroethylene, which can form phosgene when heated. Therefore, industrial workers, firemen, military personnel, and the average citizen are at increased risk for accidental or occupational exposure to phosgene. In Poland, for example, because of heavy industrialization and its proximity to densely populated areas, phosgene, along with chlorine, ammonia, and sulfur dioxide, has been identified as one of the most significant threats to the environment (5). Although it has never been scientifically verified, the estimated 50% lethal concentration over time (LCt_{50}) in man is approximately 800 ppm \times min (3200 mg/m³) for a 2-minute exposure (6).

For nearly 80 years, it has been known that phosgene has the capacity to cause severe lung injury. Exposure to phosgene may develop into fulminating and life-threatening pulmonary edema 6-24 hours after initial exposure (7,8,79). Mechanisms related to this "latency" phase have eluded investigators. Research over the past six decades has shown that direct exposure to phosgene did not cause systemic poisoning, but that its primary effects were seen in deep lung compartments (9,10). Phosgene reacts with important cellular components of biomolecules, such as sulphydryl, amine, and hydroxyl groups (11). Considering this chemical reactivity, there is little doubt that exposure has been found to directly affect type I pneumocytes (12,13), increase lavage polymorphonuclear phagocytes (14), decrease both cytochrome C oxidase and ATPase activity (15), and significantly reduce lung ATP concentrations (14). The long-term effects of phosgene inhalation are also known to eventually result in immunosuppression that may increase mortality via infection in the compromised lung (16,17).

Recent experimental work in animals has shown that phosgene produces a wide range of effects. For a review see Diller and Borak (18). Phosgene also has been found to be toxic through normal metabolic detoxification mechanisms unrelated to direct inhalation exposure. In hepatocytes, it was determined that the oxidative metabolite of chloroform, phosgene, forms adducts with phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) under hypoxic or normoxic conditions (19-21). Adduct formation could be mechanistically

important during the injury process because alveolar surfactant is largely phospholipid in content and alveolar edema causes a locally hypoxic environment (22).

Phosgene gas has been and continues to be a potentially serious and occasionally fatal substance encountered in everyday life (23). The purpose of this paper is to summarize the effects of post-exposure treatment of phosgene injury in rabbits and mice using selected clinically available drugs.

Experimental Material and Methods for Rabbit Studies

The exposure chamber was constructed from 1 cm thick Plexiglas having dimensions of 30 cm³. Exposure chamber and methodology have been described previously (24,25). As an estimation of dose, we used the product of concentration (ppm) x time (min) to standardize exposure conditions. This has been shown to be linear with short exposures (26). Phosgene, 250-400 ppm mixed with air in a 3Q cylinder, was purchased from Matheson Gas Products (East Rutherford, NJ). Phosgene concentrations were verified in a dynamic exposure system by a simple titration method. Once the phosgene concentration was determined, it was a simple matter to calculate the exposure time necessary to provide a concentration x time exposure dose of 1500 ppm-min. For example, if the phosgene concentration was analyzed to be 250 ppm then rabbit exposure duration was six minutes.

Male New Zealand white rabbits (Bunnyville Farm, Littlestown, PA) weighing 2.5-3.0 kg were maintained on Southern States rabbit formula and provided water ad libitum. Following exposure, rabbits were anesthetized with pentobarbital sodium (25 mg/kg) and given 1000 U of heparin by ear vein. Surgical procedures have been described previously (24,25). Briefly, the lungs were perfused with buffer at 40 ml/min. The chemical composition of Krebs-Henseleit buffer in mM was the following: NaCl, 83.1; KC1, 4.7; CaCl₂/2H₂O, 2.53; KH₂PO₄, 2.6; MgSO₄, 1.19; dextrose, 11.1; and NaHCO₃, 25. All lung perfusions were begun 50-60 minutes after start of exposure and were perfused initially for 10-15 minutes in a non-recirculating manner with the buffer to wash blood out of the lung. Lungs were ventilated with 5% CO₂, balance air, via a tracheostomy, using a tidal volume of 30 ml at 18 breaths/min with a Harvard animal respirator (Harvard Apparatus, Natick, MA).

Pulmonary artery pressure (Ppa), tracheal pressure (Pt), and lung weight gain (LWG) were recorded continuously using Statham P23DB pressure transducers (Statham Inst., Hato Rey, PR) and a Grass polygraph (Model 7D, Grass Inst., Quincy, MA). Lung weight changes were recorded continuously as the converse of the weight change of the perfusion reservoir, which was freely suspended from a force displacement transducer (Grass Inst., Model PT 1OC).

Aminophylline was obtained from American Regent (Shirley, NY). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental Treatment Groups

Rabbit exposure treatment groups were randomly allocated in a Latin square design. All phosgene-exposed rabbits received a cumulative dose of 1500 ppm·min that produced a consistent and reproducible edema in the isolated perfused rabbit lung model within 2-3 hours after the start of exposure (24,25,27,28).

Unexposed controls: Control rabbits were placed in the chamber and given a 10-minute exposure to room air only. This group was used in tissue analyses only.

Phosgene exposed: This group was exposed to phosgene at 1500 ppm·min as determined from earlier exposure-response experiments.

Aminophylline treatment: After exposure to phosgene and following the 10- to 15-minute washout period, aminophylline (30 mg/kg) was added to the perfusion circuit, consisting of 200 ml of KH perfusate, at 80-90 minutes after start of exposure. The average concentration of aminophylline at the beginning of drug intervention was 400 ug/ml perfusate. This was reduced to approximately 210 ug/ml after perfusate sampling of 24 ml at 90, 110, 130, and 150 minutes after start of exposure (see sample analysis below).

Isoproterenol intratracheal treatment, ISO IT: After exposure to phosgene, isoproterenol (24 µg) was mixed with 1 ml Krebs buffer and given intratracheally at the beginning of perfusion (50-60 minutes after start of phosgene exposure).

Constant isoproterenol intravascular infusion, ISO IV: After exposure to phosgene, rabbits were given a constant ISO (24 µg/min) infusion directly into the pulmonary artery perfusion line during the entire course of perfusion. ISO given in this manner was extremely toxic to the lung and caused rampant edema within 10-15 minutes after start of perfusion. Therefore, these data are not available.

Combination intravascular infusion and intratracheal bolus of isoproterenol, ISO IT+IV: After exposure to phosgene, this treatment involved the combination of treatments discussed in the two previous ISO groups.

N-acetylcysteine (IT NAC) Intratracheal Bolus Treatment: AT 45- to 60-minutes after phosgene exposure, a total of 130 mg NAC (40 mg/kg) was mixed in 1 ml Krebs-Henseleit (KH) buffer and given as an intratracheal bolus.

Perfusate Leukotriene C₄/D₄/E₄ Concentration Determination

Twenty-four-ml samples were drawn from the pulmonary vein catheter at 70, 90, 110, 130 and 150 minutes postexposure, and analyzed for 6-keto-PGF1α and the sulfidopeptide leukotrienes C₄/D₄/E₄. From this volume, 20 ml was used to analyze for arachidonate mediators. Samples were processed in the following manner: C18 SEP PAK (Waters, Milford, MA) filters were prewashed with 10 ml methanol followed by 10 ml of distilled water thereby activating the

column. Next, the 20 ml sample was filtered and eluates were washed from the filter with 5 ml methanol. These final sample products were lyophilized to near dryness and stored at -85°C prior to analysis. Arachidonic acid metabolites were measured by RIA technique using rabbit anti-LTC₄, which had an 80% cross-reactivity with LTD₄ (29). Dextran-coated charcoal was used for separation of free and bound radioligand. The sensitivity of this assay was 0.1 pmol/ml. The recovery of leukotrienes as determined by RIA after C18 SEP PAK fractionation was approximately 87 + 1% (30).

Lung Tissue cAMP Concentration Determination

At 150 minutes after exposure, left lungs were removed and freeze clamped in liquid N₂ as previously described for GSH. Frozen samples were pulverized in liquid N₂ or dry ice and 100 mg removed for analysis of cAMP. Rainen cAMP ¹²⁵I RIA kits (DuPont, Wilmington, DE) were used for analysis. These kits utilized a double-antibody RIA with a pre-reacted antibody complex. Basically, 100 µl of sample or tissue supernatant was diluted 1:5 in a supplied assay buffer. In the acetylation procedure, which was used for greater sensitivity, 150 µl of triethylamine/acetic anhydride mixture was added to the 1:5 diluted sample along with 5 µl of acetylation reagent. Following acetylation, 100 µl of ¹²⁵I tracer and 100 µl of antiserum were added. These samples were then incubated at 2°C-8°C overnight, precipitated with Na azide, centrifuged at 1200 x g, supernatant removed and radioactivity in the pellet determined in a gamma detector (Beckman Instruments, Inc.) for 1 minute. Amount of bound ¹²⁵I cAMP in sample was determined by comparison with a standard curve. The sensitivity of this assay was 100 fmol/ml.

Lung Tissue Glutathione (GSH) Determination

Immediately at the completion of perfusion the left lung was freeze-clamped in liquid N₂. GSH determination included the oxidized form (GSSG) and reduced form, GSH, and total GSH. The assay for GSH followed that of Tietze (31). Five hundred mg of lung tissue was pulverized in liquid N₂ then placed in Na phosphate buffer, pH 7.5, homogenized for 30 sec using a polytron (PT 10-3S Kinematic, Brinkman Ins. Westbury, NY), and centrifuged 5 minutes at 10,000 x g. Lung tissue proteins were precipitated with 1M perchloric acid. Supernatants were analyzed for total GSH by the catalytic conversion to GSH by glutathione reductase in the presence of 5,5' DTNB (dithiobis 2-nitrobenzoic acid). GSSG was determined in a similar manner following treatment with N-methylmalimide (NEM)(32). NEM was removed before analysis by C18 SEP PAK filtration. GSH and GSSG concentration were determined on a UV-VIS Lambda spectrophotometer (Model Lambda 4, Perkin Elmer, Norwalk, CT) at 412 and 340 nm, respectively.

Lung Tissue Thiobarbituric Acid Reactive Substances (TBARS) Determination

Treated and untreated perfused lungs were analyzed for TBARS which is believed to be an indicator of lipid peroxidation. Lung tissue, 250 mg, was homogenized on ice with 2 ml of 1.15% KCl and mixed with 4 ml of thiobarbituric acid (TBA) reagent, which consisted of 0.375% TBA in 0.25 N HCL plus 0.01% butylated hydroxytoluene. Following incubation at 100

$^{\circ}\text{C}$ for 20 min and centrifugation at 1000 x g for 10 minutes, TBARS absorbance in the supernatant was measured at 532 nm and concentration calculated using the extinction coefficient 1.56 x M-1cm-1 (33).

Protein Determination

One hundred mg of pooled lung tissue was analyzed for tissue protein concentration by the method of Lowry and co-workers [34].

Statistical Analysis

Outcome parameters of interest in this study were 1) pulmonary artery pressure, 2) lung weight gain, 3) tracheal pressure, 4) perfusate concentration of LTC₄/D₄/E₄, and 5) lung tissue TBARS, cAMP concentration, and GSH redox status. We were interested in both temporally related treatment effects and data comparisons at each time point. The design of this study in terms of sampling during perfusion times at 70, 90, 110, 130, 150, and 180 minutes after exposure, constituted a repetitive analysis. Since we were comparing treatments over time, a two-way ANOVA with repeated measures was used. Duncan's multiple range test was used for multiple comparison of means. A one-way ANOVA was used to compare tissue concentration of TBARS or glutathione. Tukey's post hoc test was used if data were significantly different (35). All data are presented as mean \pm SEM and were considered statistically significant at $p \leq 0.05$.

Experimental Material and Methods for Mouse Studies

Male CD-1 (Charles River, Wilmington, MA) mice weighing 25-30 grams were exposed whole-body to an equivalent concentration x time (cxt) amount of 32 mg/m³ phosgene for 20 min (640 mg \times min/m³). All elements of exposure were performed in an approved laboratory fume hood. Ten percent phosgene:balance N₂ (Matheson Gas Products, Baltimore, MD) was metered through a Tylan® mass flow controller (Tylan Corp., Torrance, CA) at a rate dependent on the desired concentration. This was mixed with room air and then passed through an infrared spectrometer (Miran 1A, Foxboro Co., Sharon, MA). The Miran 1A was equipped with a real-time analog output. Concentration versus time graphs were developed and the input concentration was calculated. The exposure occurred in a Plexiglas® cylinder (25 cm in height x 28 cm in diameter) with a total volume of 15.8 l at a flow rate of 20 l/min. Exposure to phosgene was for 20 min followed by a 5-min room air washout. Out-flowing gas from the chamber was passed through a second Miran 1A unit to determine the concentration of phosgene exiting the chamber. Effluent from the hood was passed first through an M18 filter and then through standard activated charcoal fume hood filters. Mice were exposed in two groups of forty each to identical phosgene concentrations.

Administration of Ibuprofen

Ibuprofen (Sigma Chemical Co. St Louis, MO) concentrations were determined from the published LD₅₀ for mice, which is 495 mg/kg for an intraperitoneal injection [36]. Dosing times were determined based on the t_{1/2} of IBU, which is about 2-4 h, and on previous work done in

this laboratory using rats (37). IBU was mixed in saline 42 mg/ml and pH was adjusted to 7.5 with 300 μ l of 0.25 M HCl; the IBU was then sterile filtered using a 0.45 μ filter cup. IBU was administered in a total volume of 500 μ l in the following doses: 0 (saline), 3, 9, 15, and 21 mg per mouse. Sixteen mice per drug dose were used. Immediately following exposure, mice were injected with IBU i.p. in the lower right abdominal quadrant. Five hours later a second i.p. injection was given to these same mice in the lower left abdominal quadrant, but at only one half the original dose 0, 1.5, 4.5, and 7.5. Mice exposed and then injected with 21 mg IBU experienced a high mortality rate within several hours of the injection. To eliminate any confounding effects of phosgene exposure with IBU toxicity at this dose, data from these mice were eliminated from analysis.

Tissue Collection Procedure

Mice were observed for 12 h from the start of exposure and again at the 24-h time point after the start of exposure. Mice that expired within 12 h were weighed immediately and necropsied for their lung tissue. The left lung was weighed and placed in a tared planchete for wet/dry weight ratios. Lungs for the wet/dry ratio (WW/DW) were placed in an oven at 100°C and reweighed eight days later for dry weight. The entire right lung was quickly frozen in liquid nitrogen and stored under nitrogen at -80°C for future biochemical measurements. All lung weight gravimetric data are from mice that expired up to 12 h after start of exposure.

Tissue Biochemical Assays

Because the mass of individual mouse lungs was small, we pooled lung tissue within the same drug treatment groups. Three to four mouse lungs were pooled for each treatment dose per assay. This enabled us to adequately perform assays in duplicate. All tissue assays were performed on pooled mouse lung tissue ground to a fine powder under liquid nitrogen.

TBARS Concentration Determination for Mice Tissue

Lung tissues from mice were analyzed for TBARS, an indicator of lipid peroxidation. One hundred mg of pooled lung tissue was homogenized in 900 μ l of 1.15% cold KCl solution along with 15 μ l of 0.3% butylated hydroxytoluene (BHT) in ethanol. Four hundred μ l of this 10% homogenate was mixed with 3 ml of trichloroacetic acid (15% w/v in 0.25 N HCl) and 1 ml of thiobarbituric acid solution (0.37% w/v in 0.25 N HCl) in stoppered 10 ml test tubes. These tubes were vortexed and centrifuged for 15 min at 2000 x g to pellet the protein. Supernatants were removed and boiled for 1 h at 100°C and then chilled on ice. After cooling, chromagen was removed using 4 ml of 4-butanol, vortexed, and centrifuged at 1000 x g for 20 min. The organic phase was collected and absorbance read at 532 nm against a sample blank in a UV-VIS spectrophotometer Model 2101-PC (Shimadzu Scientific Ins., Inc., Columbia, MD). TBARS concentration was calculated using an extinction coefficient of 1.56 x 105 M⁻¹cm⁻¹. The assay described above combined the techniques from several sources (33,38,39). To check the accuracy of the assay, standards using 1,1,3,3, tetraethylpropane processed in a similar manner were run. A greater than 90% recovery of TBARS was observed using the combined procedures described above.

Nonprotein Sulphydryl (NPSH) Concentration Determination

As an estimate of protective thiol concentrations, namely, GSH, nonprotein bound sulphydryls were measured using the method outlined by Sedlak and Lindsay (40). Briefly, 75 mg of pooled frozen lung tissue was homogenized in 0.02 M EDTA and then mixed with distilled water and 15% trichloroacetic acid (TCA). Next, these homogenates were mixed with 0.4 M tris buffer and 0.2 M EDTA, pH 8.9, and 100 µl of DTNB and slowly shaken at room temperature for 15 min. This solution was then centrifuged at 3000 x g for 15 min. Absorbance values of supernatants were recorded at 412 nm using a UV-VIS spectrophotometer. NPSH concentration was calculated using the published molar extinction coefficient of $13.1 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ (40).

Protein Determination

Fifty mg of pooled lung tissue was analyzed for tissue protein concentration by the method of Lowry and co-workers (34). Both nonprotein sulphydryls and MDA were standardized using lung tissue protein concentrations.

Statistical Analysis for Mice Studies

Comparisons of lung weight parameters or tissue biochemical measurements between exposed drug-treated mice and exposed saline-treated mice were analyzed by one-tailed Student's t-test. Survival was statistically evaluated using the Chi square (χ^2) distribution. Calculations of odds ratios for survival were performed according to Kahn (41, see footnote in Table 3). Data were considered statistically different at a significance level of $p \leq 0.05$.

Results and Discussion

Phosgene is a highly toxic gas that is capable producing widespread tissue destruction and cell death. Because of its insoluble nature, phosgene attacks the deeper regions of the lung where it can alter the GSH redox enzymes (42). Furthermore, phosgene has been shown to increase lipid peroxidation, cause the release of sulfidopeptide leukotrienes, alter important cell ultrastructures, and react directly with lung surfactant (22,24,27,43). In addition, it has been shown that phosgene can form adducts with renal microsomal phospholipid polar heads (19). In the lung, the end result of these disturbances is an increase in susceptibility to interstitial edema followed by alveolar flooding that can be potentially fatal for exposed individuals (23,44,79). With these particular outcomes in mind, these studies were designed to investigate the efficacy of post-treatment with clinically available compounds on mice exposed *in vivo* to a lethal dose of phosgene.

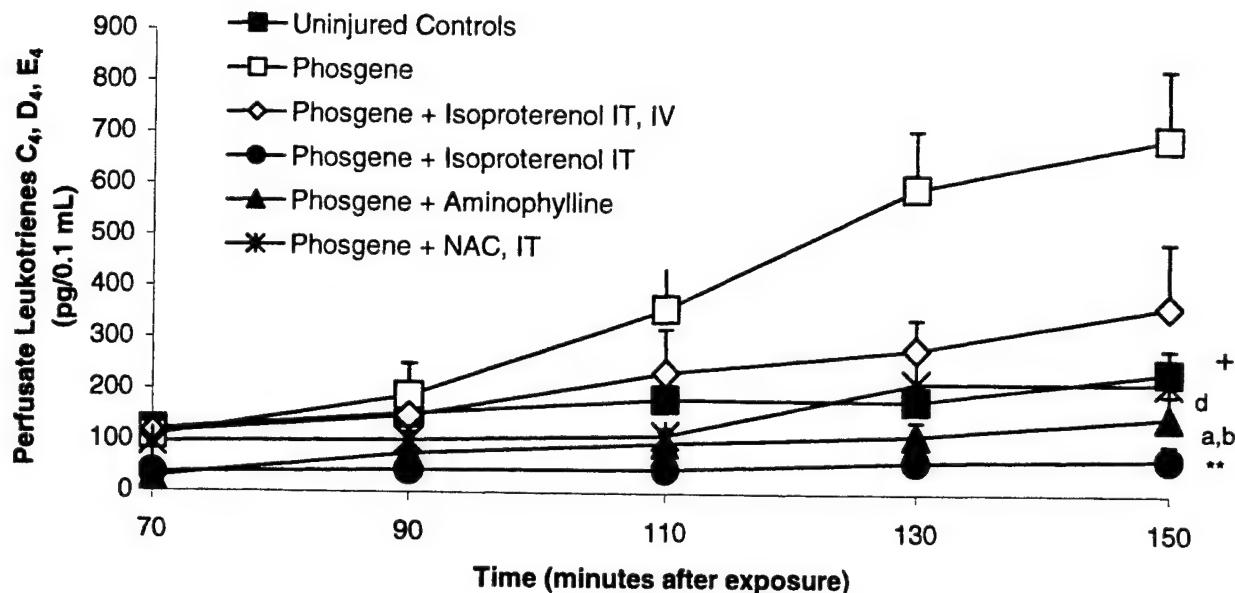


Figure 1. The effect of phosgene exposure on rabbit lung perfusate levels of leukotrienes 70-150 minutes after exposure. Treatment x time effects: (+) Controls significantly < phosgene, $p \leq 0.017$; (a) AMIN < controls, $p \leq 0.039$; (b) AMIN < phosgene, $p \leq 0.016$; (d) (IT) NAC < phosgene, $p \leq 0.01$; and (**) ISO IT < control, $p \leq 0.042$. Timing for postexposure therapies: ISO (IT+IV) constant infusion of 24 $\mu\text{g}/\text{min}$ from 50-150 minutes, ISO given intratracheally (IT) between 50-60 minutes, AMIN (30 mg/kg) added to perfusate between 80-90 minutes, NAC (40 mg/kg) given (IT) between 45-60 minutes after exposure.

Exposure to phosgene significantly increased the release of sulfidopeptide leukotrienes into the perfusate over time when compared with most groups except the ISO-treated and exposed groups (Figure 1). Although the difference between phosgene and the ISO groups appears to be large especially at 150 min, small sample size for ISO (IT), $n=2$, probably contributed to the failure of statistical significance. The reduction or reduced presence of leukotrienes is crucial in tissue injury because leukotrienes may be important especially in phosgene-induced lung injury. Novel experiments performed in guinea pigs by Trethewie in 1947 indicated that SRS-A, now known as the sulfidopeptide leukotrienes, was released into the perfusate from the vascular compartment in lungs after phosgene exposure (45). It has been shown that the administration of LTD₄ caused an increase in lung weight gain in the isolated buffer-perfused rabbit lung (46). Reduction of perfusate leukotrienes seen in the ISO- and AMIN-treated groups is consistent with the inhibition of sulfidopeptide leukotrienes after administration of dibutyryl cAMP (27). Both ISO and AMIN increase cAMP levels via different mechanisms; ISO, as a β -adrenergic compound that causes adenylate cyclase to catalyze the breakdown of ATP and AMIN via its phosphodiesterase activity. AMIN could also protect against lung injury since leukotrienes appear to be important in the pathophysiological process (46). In fact, there have been studies that demonstrated that AMIN decreased lung mast cell leukotriene production and that therapeutic concentrations of theophylline inhibited PMN superoxide anion generation and reduced calcium mobilization (47,48). Intratracheal treatment with NAC caused a significant treatment x time reduction in perfusate leukotriene concentrations. The mechanism of this reduction is not known, but it may be related to NAC's

capacity to reduce the effect of toxic free radical processes through the up-regulation of the glutathione redox cycle (Table 2).

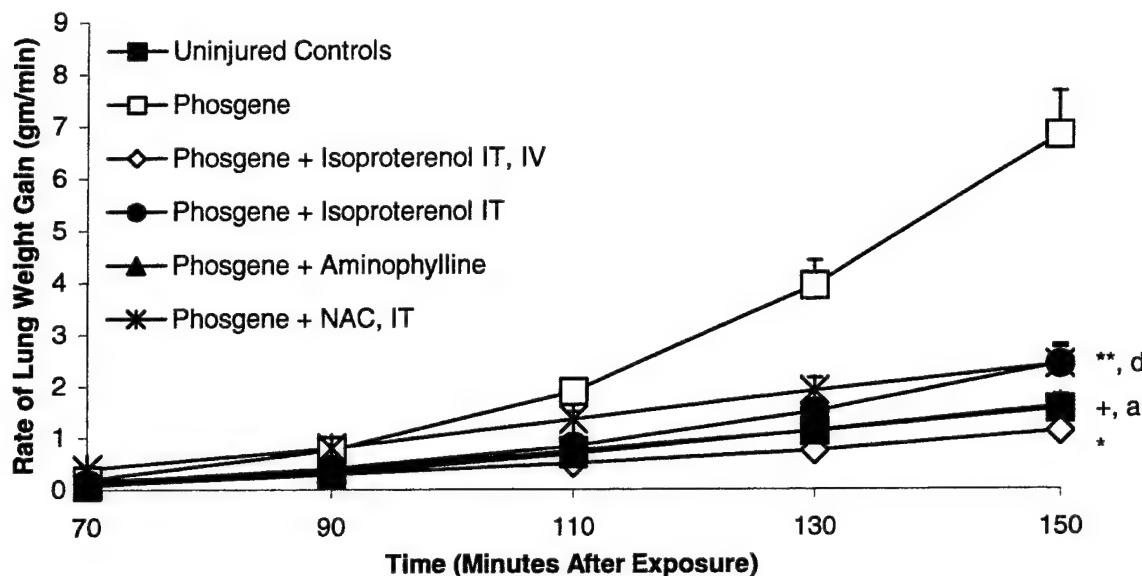


Figure 2. The effect of phosgene on pulmonary edema formation (lung weight gain) in rabbit lungs 70-150 minutes after exposure. Treatment x time effects: (+) controls<phosgene, $p \leq 0.0001$; (a) AMIN <phosgene, $p \leq 0.0006$; (d) (IT) NAC<phosgene, $p \leq 0.05$; (*) ISO IT+IV<phosgene, $p \leq 0.0006$; and (**) ISO IT<phosgene, $p \leq 0.008$. Timing for postexposure therapies: ISO (IT+IV) constant infusion of 24 $\mu\text{g}/\text{min}$ from 50-150 minutes, ISO given intratracheally (IT) between 50-60 minutes, AMIN (30 mg/kg) added to perfusate between 80-90 minutes, NAC (40 mg/kg) given (IT) between 45-60 minutes after exposure.

Compared with uninjured air-exposed rabbits, 1500 ppm-min (6000 mg-min/m^3) phosgene caused a profound increase in the rate of lung weight gain over time (Figure 2). At 150 minutes the rate for phosgene-exposed rabbits was nearly 35-fold higher than time point matched controls. Post-treatment of phosgene-exposed rabbits with ISO was very effective in ameliorating pulmonary toxicity as measured by edema formation (LWG). A reduction in permeability was reported by other investigators using ISO treatment in other forms of acute injury (49,50). Infusion of pharmacological doses of ISO or AMIN decreased lung permeability and pulmonary hypertension caused by lipid hydroperoxide infusion (51). AMIN significantly reduced the rate of LWG from 130-150 compared with phosgene ($p \leq 0.01$). Postexposure (IT) treatment with NAC caused a slightly significantly lower rate of edema formation when

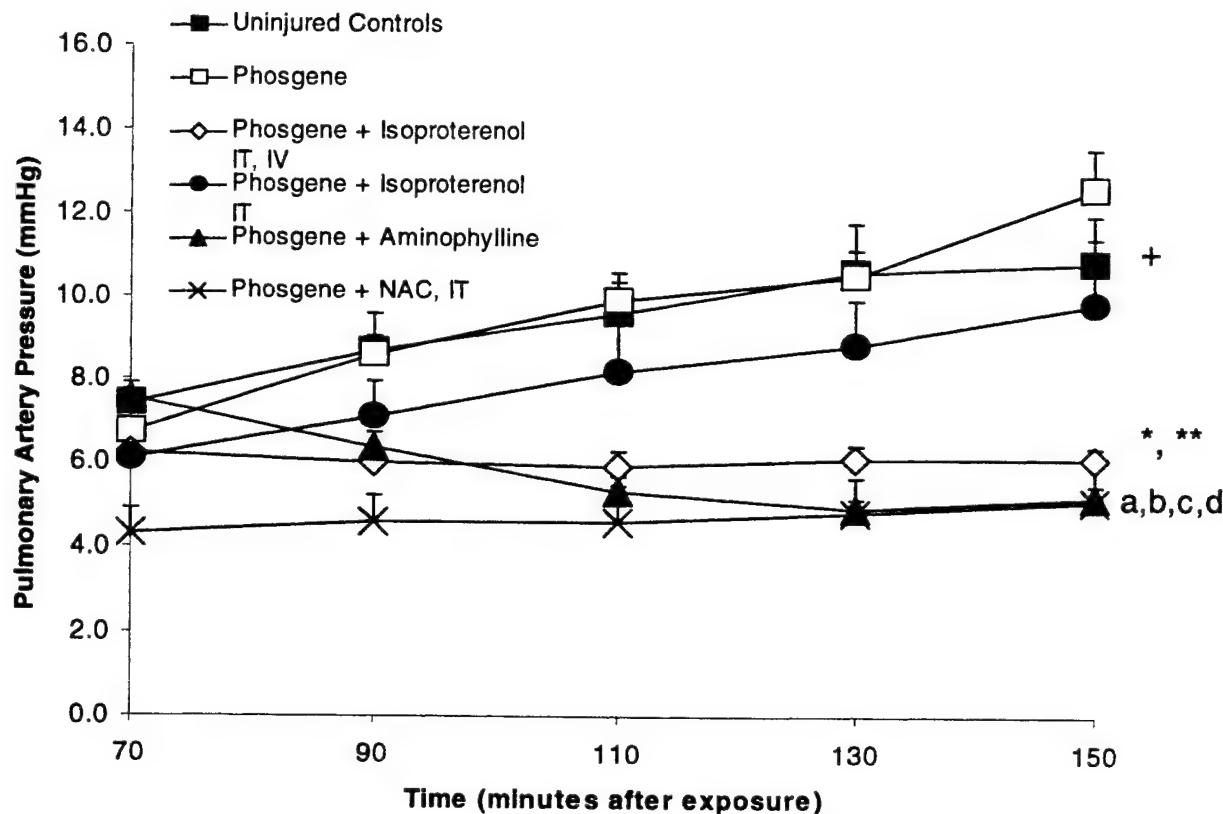


Figure 3. The effect of phosgene exposure on pulmonary artery pressure in rabbit lungs 70-150 minutes after exposure. Treatment x time effects: (+) Controls<phosgene, $p \leq 0.02$; (a) AMIN< control, $p \leq 0.011$; (b) AMIN<phosgene, $p \leq 0.0001$; (c) N-acetylcysteine<control, $p \leq 0.02$; (d) (IT) NAC<phosgene, $p \leq 0.001$; (*) ISO IT+IV<control, $p \leq 0.031$; and (**) ISO IT+IV<phosgene, $p \leq 0.0003$. Timing for postexposure therapies: ISO (IT+IV) constant infusion of 24 $\mu\text{g}/\text{min}$ from 50-150 minutes, ISO given intratracheally (IT) between 50-60 minutes, AMIN (30 mg/kg) added to perfusate between 80-90 minutes, NAC (40 mg/kg) given (IT) between 45-60 minutes after exposure.

analyzing treatment x time effects compared with phosgene. While (IT) NAC's effect on LWG is largely unexplained, there may be some connection between NAC's capacity to reduce lipid peroxidation, as measured by TBARS, as well as its effect on the glutathione redox cycle (Table 1). The effect on both processes by NAC may increase membrane stability in a phosgene-induced toxic environment thereby preventing or at least inhibiting transudation of fluid into the air space.

In Figure 3, the effects of postexposure therapies on pulmonary artery pressure are shown. Mean Ppa measured in the lungs from uninjured control and phosgene-exposed rabbits was slightly increased between 70 and 150 minutes. However, Ppa in control lungs was slightly, but significantly, lower than phosgene at 150 minutes after exposure, $p \leq 0.01$. ISO was

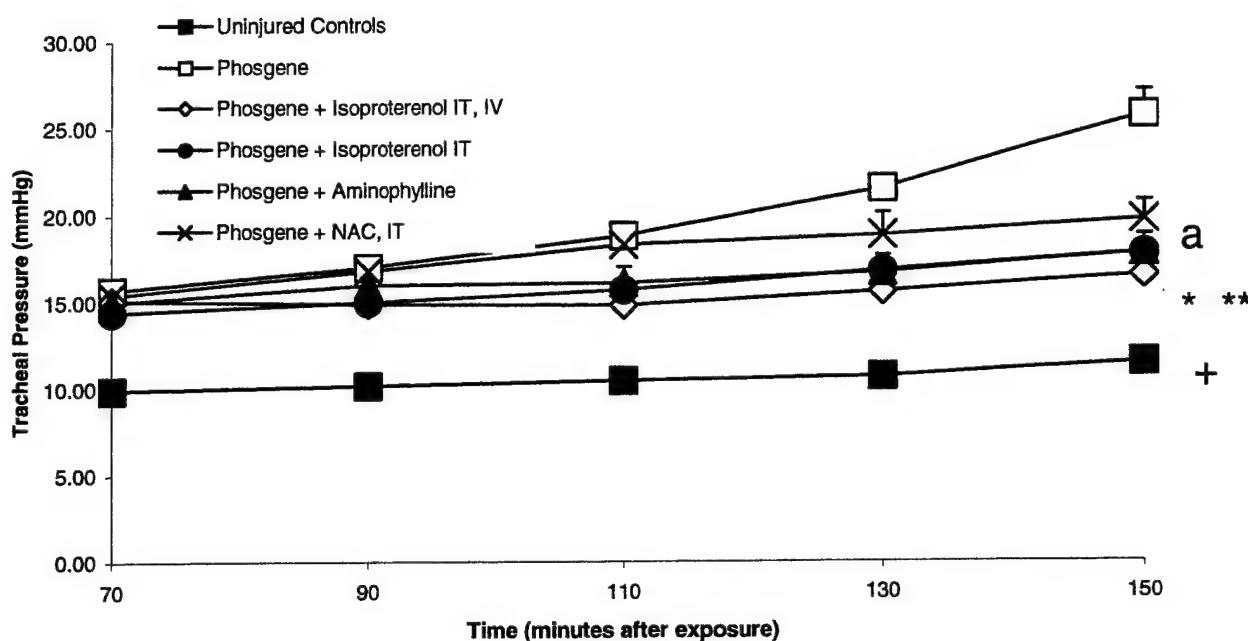


Figure 4. The effect of phosgene exposure on tracheal pressure in rabbits 70-150 minutes after exposure. Treatment x time effects: Controls< all other groups, $p\leq 0.001$; (a) AMIN<phosgene, $p\leq 0.004$, (*) ISO IT+IV<phosgene, $p\leq 0.001$; and (**) ISO IT<phosgene, $p\leq 0.004$. Timing for post-exposure therapies: ISO (IT+IV) constant infusion of 24 μ g/min from 50-150 minutes, ISO given intratracheally (IT) between 50-60 minutes, AMIN (30 mg/kg) added to perfusate between 80-90 minutes, NAC (40 mg/kg) given (IT) between 45-60 minutes after exposure.

specific for reducing phosgene-induced increases in Ppa since it also reduced Ppa below control levels. Ppa in ISO (IT+IV) post-treated rabbits was reduced over time compared with both phosgene-exposed rabbits and uninjured controls, whereas Ppa in the ISO (IT) treatment was increased to a greater extent than in the ISO (IT+IV) treatment. Presumably, these results are due to the well-known vasodilation effects of ISO. These data suggest that the β_2 adrenergic receptors on the capillary endothelial membrane are intact, resulting in lower Ppa. In contrast, since exposure to phosgene affects the epithelial membrane to a greater extent, ISO (IT) treatment may be less effective, resulting in a vasodilatory effect not as pronounced as that seen with the ISO (IT+IV) treatment. There was a significant treatment x time effect of AMIN on lung Ppa when compared with phosgene over time. This was particularly the case starting from 90 min to the end of the perfusion. Intratracheal administration of NAC caused a small but statistically significant decrease in Ppa in comparison with the phosgene and control groups.

Figure 4 depicts the effects of phosgene exposure and treatments on tracheal pressure (Pt). Phosgene exposure also caused increased tracheal pressure, which occurred prior to increased lung weight gain, suggesting bronchoconstriction rather than decreased lung compliance as the cause of the increase in tracheal pressure. Mean Pt in control rabbit lungs was significantly lower than in the phosgene-exposed group, which increased 63% from 70 to 150 minutes vs 15% for controls. While ISO (IT+IV) could not reduce Pt to uninjured control levels, it significantly prevented the rapid rise in mean Pt measured in phosgene-exposed rabbits from 130-150 minutes ($p\leq 0.01$). ISO (IT) post-treatment did not reduce mean Pt to uninjured control

levels from 70-150 min, but it did prevent increasing bronchoconstriction observed in phosgene-exposed rabbits. Pt with ISO (IT) post-treatment rose only from 14.4 ± 0.6 to 17.8 ± 1.1 mmHg during this same time interval. Although this increase is not as great as that observed in phosgene-exposed rabbits, it is significantly greater than in uninjured controls ($p \leq 0.01$) from 70-150 minutes. Comparison of (IT) with (IT+IV) showed that treatment x time effects on Pt were not different. Post-treatment with AMIN attenuated the increase in Pt, rising only 19% from 70-150 minutes. The effect of AMIN treatment was particularly evident when compared with phosgene from 130-150 minutes. Overall, the increase in tracheal pressure was not effectively blocked by (IT) administration of NAC (Figure 4). This phenomenon, which does not seem to be prevented by antioxidant treatment, may be important in the pathophysiology of phosgene-induced lung injury since there are several anecdotal reports of wheezing in cases of human phosgene exposure (52).

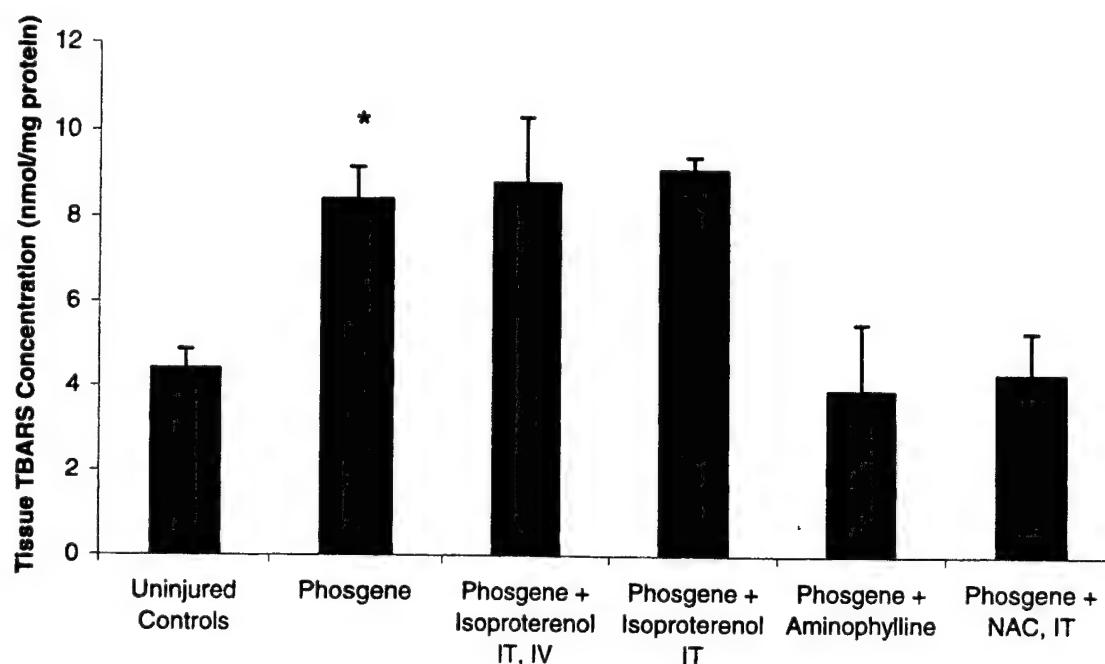


Figure 5. The effect of phosgene on rabbit lung tissue lipid peroxidation as measured by TBARS at 150 minutes after exposure. Phosgene is significantly higher than controls, $p \leq 0.004$; AMIN, $p \leq 0.042$; and (IT) NAC, $p \leq 0.05$. Timing for postexposure therapies: ISO (IT+IV) constant infusion of 24 $\mu\text{g}/\text{min}$ from 50-150 minutes, ISO given intratracheally (IT) between 50-60 minutes, AMIN (30 mg/kg) added to perfusate between 80-90 minutes, NAC (40 mg/kg) given (IT) between 45-60 minutes after exposure.

The effects of phosgene-exposure and therapeutic agents at 150 minutes after exposure on lipid peroxidation as measured by TBARS in lung tissue homogenates are shown in Figure 5. Exposure to phosgene markedly enhanced the formation of lipid peroxidation by products by 89% when compared with uninjured controls. Both ISO treatment groups had no effect on the formation of TBARS products in lung tissue and were, in fact, equivalent in raw value when compared with phosgene-exposed rabbits. Lung tissue TBARS concentration following aminophylline administration was comparable to controls, but significantly reduced, by 53%,

when compared with phosgene-exposed rabbits at 150 minutes. NAC treatment (IT) caused a slight but significantly lower TBARS concentration versus that measured for phosgene.

Table 1. Lung tissue cAMP concentration. Samples were analyzed at 150 minutes after exposure. Data are expressed as pmols/gram wet lung weight (mean \pm SEM).

Baseline controls (n=5)	Uninjured controls (n=12)	Phosgene (n=10)	ISO(IT+IV) (n=3)	ISO(IT) (n=6)	AMIN (n=4)
19.9 \pm 3.5	15.3 \pm 1.8	6.2 \pm 0.9*	10.3 \pm 5**	9.5 \pm 2.6**	9.8 \pm 1.1**

* Phosgene-exposed is significantly lower than uninjured controls P \leq 0.0001.

**Treated groups are significantly different from phosgene-exposed, P \leq 0.05 (one-tailed t-test).

The data in Table 1 shows that phosgene exposure lowers lung tissue cAMP concentration measured at 150 minutes after exposure. This may be an indication of phosgene-enhanced PDE activity. PDE has been shown to be present in mouse alveolar type I and type II epithelial cells (53). In addition, it has been demonstrated that increases in free calcium can lead to enhanced PDE activity, leading to a decrease in cyclic nucleotide content (54). Previous work from this institute has shown that phosgene exposure caused a rapid, <45 sec, 2- to >5-fold increase in intracellular free calcium concentration in sheep pulmonary artery endothelial cells (55). Taken together, one may speculate that AMIN may ameliorate the PDE-enhancing effects of phosgene by maintaining appropriate cAMP levels via its anti-PDE action. Increased cAMP levels are necessary to keep smooth muscle cell tight junctions intact to prevent or impede interstitial and/or alveolar edema (Figure 2).

Post-treatment with ISO helped to maintain tissue levels of cAMP. While not equivalent to uninjured control values, there was a significant amount of cAMP in ISO-treated tissue to keep tight junctions in a relaxed state and possibly ameliorate massive edema seen in phosgene-exposed rabbits. ISO may also work through a newly postulated mechanism. It has recently been shown in cell-free experiments that ISO has the capacity to act directly as an antioxidant by reducing AOH and O₂- reactive species (56).

One postulated mechanism for changes in permeability with ISO treatment is that it increases cAMP-induced relaxation of tight junctions between epithelial and endothelial cells, thereby reducing fluid flux (57,58). In the present study, elevated leukotriene concentrations measured after ISO (IT+IV) post-treatment, but not ISO (IT) post-treatment (Figure 1) suggest that the presence of ISO on the vascular side may be causing release of these mediators. This may be one reason why ISO (IV) was completely ineffective (data not available) and why the addition of ISO (IT) helps to stabilize the damaging effects.

The effects of phosgene exposure on calcium translocation, although not measured in this study, may be partly responsible for the protection seen with ISO treatment. As mentioned above, previous work has shown that phosgene exposure caused an increase in intracellular free calcium concentration in cultured lung endothelial cells (55). β -receptor stimulation has been shown to affect intracellular calcium levels (59,60). Activation of PLA₂ calcium could also give rise to arachidonic acid metabolites such as the leukotrienes or prostaglandins. It is unclear what effect β -adrenergic receptor stimulation has on membrane calcium levels. In this study, we did observe a significant decrease in leukotriene concentration in ISO (IT)-treated animals even when compared with the control group (Figure 1). Thus, the observed effect of lowered Ppa, decreased LWG, and reduced LTC₄/D₄/E₄ may result from decreased calcium mobilization.

The data in Table 1 may partially explain the effect of AMIN treatment of phosgene-exposed lungs on pulmonary artery pressure (Figure 3). From Table 1 it can be seen that exposure to phosgene may have caused an increase in PDE activity thereby lowering lung tissue cAMP concentration through hydrolysis to 5'-AMP. Also from Table 2 treatment of phosgene-exposed lungs with AMIN-inhibited PDE with the result being a cAMP concentration slightly below that of controls but significantly higher than phosgene alone. Therefore, we could speculate that the low Ppa observed in Figure 3 could be the additive effects of the vasodilatory capacity of increased cAMP levels and those of 5'-AMP which can be hydrolyzed further to form adenosine a potent vasodilator in many vascular beds (61). In summary, the major results from this study are that effective treatment of phosgene-induced acute lung injury is possible with AMIN 80-90 minutes after exposure to a lethal dose of phosgene. AMIN may protect against oxidant lung injury by the combination of the following: a direct antipermeability effect, inhibition of permeability enhancing sulfidopeptide leukotrienes, by direct or indirect antioxidant action, maintaining a cAMP concentration required to keep cellular tight junctions intact, and possibly through vasodilatory mechanisms (62). These observations suggest that AMIN or other drugs that increase cAMP may be useful for the treatment of phosgene or other oxidant gas exposures in man.

AMIN at the concentration used in these experiments reduces the amount of phosphodiesterase (PDE) available for the hydrolysis of cAMP to 5'-adenosine monophosphate (5'-AMP) and consequently induces accumulation of cAMP (63). We and other investigators have shown that drugs that increase cAMP have protective effects in several models of lung injury and in cultured endothelial cell monolayers. This has been reviewed previously (64). Pretreatment with drugs that up-regulate cAMP protect in several lung injury models, including peroxide vascular injury, endotoxin lung injury, acid aspiration lung injury and phosgene-induced acute lung injury (25,47,49,64).

Phosgene lung injury is a form of alveolar oxidant injury and it appears that drugs like dibutyryl cAMP are more effective when administered intratracheally rather than intravascularly (27). In the present experiment, vascular AMIN administration after phosgene exposure was effective in preventing edema formation (Figure 2). Post-treatment with AMINO also decreased Ppa and Pt (Figures. 3-4). This effect was observed also in AMIN-treated uninjured controls (25).

The mechanisms involved in cAMP-mediated protection appear to be multifactorial (64). The best characterized of these is the prevention or reversal of endothelial cell contraction in various injury models and in cell monolayers (*ibid*). This appears to be caused by cAMP inhibition of the phosphorylation of myosin light chain kinase (65). Cyclic AMP can also protect against phosgene-induced lung injury by other novel mechanisms. The antioxidant effects of cAMP are more clearly seen with dbcAMP, which significantly maintains glutathione reduction after phosgene exposure and prevents phosgene-induced increases in TBARS (27). Administration of AMIN similarly prevents phosgene-induced TBARS formation and tends to preserve the glutathione redox state, suggesting a common protection mechanism (Table 1). This direct or indirect antioxidant effect may explain the inhibition of peptide leukotriene production since similar results occurred after IT administration of the antioxidant NAC (28).

The effect of (IT) NAC on lung tissue cAMP concentration was not measured in this study. However, we did find that the intratracheal (IT) postexposure administration of NAC in phosgene-exposed rabbits was effective in preventing edema formation, leukotriene production, lipid peroxidation, and may preserve the normal glutathione redox state (Table 2). The effectiveness of IT administration suggests that the site of injury and action of NAC was in the deep airway compartments.

In Table 2 the effects of phosgene and post-treatment therapies on the lung tissue glutathione redox system at 150 minutes postexposure are shown. The unexpected changes in glutathione redox state in the control lungs, not exposed to phosgene, but perfused for 150 minutes with K-H buffer, suggest that blood-derived antioxidants may be important in the maintenance of lung glutathione redox state. K-H buffer contains the normal ionic composition of plasma but does not contain the normal plasma antioxidants. It seems possible that these antioxidants may play an important role in the balance between endogenous oxidant production and detoxification and could be important in the mechanism of lung reperfusion injury (66).

Phosgene significantly decreased the reduced form of GSH, increased the oxidized form (GSSG), decreased the GSH/GSSG protective ratio, and dropped the % of total as reduced GSH to 58%, which was 41% lower than controls. Post-treatment with ISO significantly increases the reduced form of GSH, GSH/GSSG ratio, and % of total as reduced GSH (Table 2). We do not know the mechanisms responsible for this discrepancy and can hypothesize that ISO may up-regulate GSH-enhancing enzymes in the GSH redox cycle. Taking into account permeability data (Figure 2) and vascular pressure data (Figure 3), it is apparent that in this lung injury model the effectiveness of β -adrenergic stimulation is due to its capacity to lower vascular pressure, inhibit leukotriene release (Figure 1), and to enhance or maintain lung tissue GSH levels. It should also be stated that β -adrenergic stimulation leads to increased surfactant secretion in Type II epithelial cells that could also function as a source of protection against phosgene (67).

Post-treatment with AMIN caused no significant changes in the glutathione redox system, although treatment did enhance the % of total as reduced GSH 22% higher than that measured for phosgene.

The mechanism of (IT) NAC protection and inhibition of leukotriene synthesis most likely relates to the ability of this drug to preserve glutathione redox state as shown in Table 1. GSH, GSH/GSSG ratio, and % reduced GSH are significantly higher, and GSSG is lower, in (IT) NAC post-treated rabbits compared with phosgene. However, we do believe that our GSSG concentrations are partially enhanced by the act of perfusion itself since in effect we are studying with a failing organ. We assume that this effect is consistent across all treatment groups. This hypothesis was supported by the effects of NAC treatment on TBARS formation (Figure. 5). It has been demonstrated in rats challenged with *E. coli* that treatment with NAC prevented increases in lipid peroxidation as measured by TBARS formation (68). It has also been suggested that NAC can function as a direct antioxidant (69); this could partially explain our observations if the toxic oxidants responsible for phosgene injury were endogenously produced. It seems possible that phosgene-induced lung injury may be caused by endogenous production of reactive oxygen metabolites after inactivation of the major water-soluble antioxidant glutathione. This hypothesis could explain the significantly lower levels of GSSG in the phosgene-exposed animals treated with NAC. The protective mechanism of (IT) NAC appears to involve (1) protection of glutathione redox state and (2) possibly NAC's direct antioxidant effects. Since NAC is clinically available for human aerosol treatment it may be useful in the treatment of human exposures to phosgene or other oxidant gases.

If we consider all the data that make up Figures 2-5 and Table 2, we can arrive at some relative association between pulmonary edema formation (LWG) and the variables of interest for phosgene-treated versus control rabbits. We calculated the relative effects of leukotrienes, Ppa, Pt, GSH, and TBARS on pulmonary edema as measured by LWG using a multiple regression analysis. It appears that there is a strong association between LWG and leukotrienes and the GSH/GSSG ratio such that

$$\text{LWG} = -6.78 + 0.8 \text{ leukotrienes} + 0.53 \text{ Ppa} + 0.61 \text{ GSH/GSSG} - 0.45 \text{ TBARS.}$$

Studies With Ibuprofen (IBU)

In the present study, it was observed that IBU significantly enhanced the 12-h survival of mice given either the 9/4.5 mg IBU or 15/7.5 mg IBU/mouse dose. This was clearly supported by the calculated survival odds ratios that indicated that mice given these doses after exposure to phosgene have 5 and 13 times, respectively, the survival compared with phosgene-exposed saline-treated mice. At 24 h the survival odds ratio is 3.5 for both dosing groups (Table 3).

Table 2. Tissue glutathione: tissue analysis at 150 minutes postexposure time. Data are expressed as nmol/mg protein (group mean + SEM). Data analyzed by one-tailed t-test.

	<u>Total GSH</u>	<u>Red GSH</u>	<u>GSSG</u>	<u>GSH/GSSG</u>	<u>% Tot as Red GSH</u>
BASELINE CONTROL (n=5)	6.7 ± 0.8	5.7 ± 0.8	0.47 ± 0.1	14.0 ± 3.2	92 ± 4.4
UNINJURED CONTROL (n=11)	5.0 ± 0.3	3.6 ± 0.3^a	0.7 ± 0.1^b	6.1 ± 0.9^c	82 ± 4.3^d
PHOSGENE (n=13)	4.0 ± 0.3	1.9 ± 0.3	1.1 ± 0.1	2.0 ± 0.3	58 ± 6.3
ISOPROTERENOL (IT,IV) (n=6)	8.4 ± 2.5	6.2 ± 1.7^e	1.1 ± 0.4	5.9 ± 0.8^f	85 ± 1.7^g
ISOPROTERENOL (IT) (n=3)	5.0 ± 0.4^h	3.0 ± 0.4ⁱ	1.0 ± 0.1	3.3 ± 0.6^j	75 ± 2.5^k
AMINOPHYLLINE (n=4)	4.2 ± 1.4	2.4 ± 1.0	0.9 ± 0.2	2.6 ± 0.6	71 ± 3.9
N-ACETYL- CYSTEINE (IT) (n=5)	3.8 ± 0.7	3.5 ± 0.7	0.13 ± 0.01^l	28.0 ± 6.5^m	95 ± 1.5ⁿ

- a. Uninjured controls different from baseline, $p \leq 0.007$, and phosgene-exposed, $p \leq 0.008$.
- b. Uninjured controls different from baseline, $p \leq 0.054$, and phosgene-exposed, $p \leq 0.003$.
- c. Uninjured controls different from baseline, $p \leq 0.003$, and phosgene-exposed, $p \leq 0.0001$.
- d. Uninjured controls different from baseline, $p \leq 0.007$, and phosgene-exposed, $p \leq 0.004$.
- e. ISO (IT+IV) different from uninjured controls, $p \leq 0.012$, phosgene-exposed, $p \leq 0.0003$, and ISO (IT), $p \leq 0.017$
- f. ISO (IT+IV) different from phosgene-exposed, $p \leq 0.0002$, and ISO (IT), $p \leq 0.021$.
- g. ISO (IT+IV) different from ISO (IT), $p \leq 0.017$.
- h. ISO (IT) different from controls, $p \leq 0.009$, phosgene-exposed, $p \leq 0.002$, and ISO (IT+IV), $p \leq 0.04$.
- i. ISO (IT) different from phosgene-exposed, $p \leq 0.3$, and ISO (IT+IV), $p \leq 0.017$.
- j. ISO (IT) different from uninjured controls, $p \leq 0.023$; phosgene-exposed, $p \leq 0.031$; and ISO IT+IV, $p \leq 0.021$.
- k. ISO (IT) different from phosgene-exposed, $p \leq 0.048$, and ISO (IT+IV), $p \leq 0.018$.
- l. NAC (IT) different from controls, $p \leq 0.003$, and phosgene-exposed, $p \leq 0.005$.
- m. NAC (IT) different from controls and phosgene-exposed, $p \leq 0.0001$.
- n. NAC (IT) different from controls, $p \leq 0.028$, and phosgene-exposed, $p \leq 0.0012$.

Increased survival at 12 h for both the 9/4.5 and 15/7.5 mg IBU-treated mice cannot be explained clearly from the results of tissue TBARS and NPSH. TBARS from the lung tissue of mice treated with 15/7.5 IBU was equivalent to that measured in the exposed and saline-treated mice, whereas TBARS was significantly lower in the exposed and 9/4.5 IBU-treated group (Table 4). Although Kennedy et al. have shown that IBU significantly reduces TBARS formation (70), the data described herein indicated that lipid peroxidation may not be a condition for survival in this drug treatment model. The same could almost be said for glutathione status as measured by the presence of NPSH. It was clearly demonstrated that NPSH were significantly greater for all IBU doses compared with phosgene-exposed and saline-treated mice (Table 4). However, although the survival for exposed and 3/1.5 mg IBU-treated mice was nearly double that of exposed and saline-treated mice at 12 h (25% vs 44%), it was not a significant change. Conditions for survival in this exposure model apparently are not as strongly related to the presence of lipid peroxidation as they may be to the presence of an intact glutathione-regulated redox state. Since the limitation of this study, i.e., lack of sufficient lung tissue, did not allow for extensive biochemical analysis, the reasons for increased survival remain speculative. Protection with IBU, shown by Kennedy et al. (70), may be due to its capacity to scavenge both the highly toxic ·OH radical and iron. Both of these are very important cofactors in cellular and tissue injury (71). Moreover, work done by Adams et al. indicated that in single dose toxicity studies, IBU causes labored respiration and sedation in rodents (72).

In the present study, a sedative effect of approximately one hour was observed after both IBU administrations. Sedation, which can decrease blood flow, may be beneficial. It has been demonstrated that IBU can lower cardiac output in dogs, which would lead to less blood flow to the lung and less alveolar edema formation (73). IBU has also been found to inhibit leukocyte migration (74). Indeed, Ghio et al. have shown that the presence of leukocytes in the lung may be responsible, in part, for edema formation in rats exposed to phosgene (75). Mice can be physically active even after exposure to an irritating and toxic gas like phosgene. Lung injury may be exacerbated by increased activity. This has been observed in rats exposed to a similarly acting edemagenic gas (76). In the present study, it may be that partial sedation, shallow breathing patterns, decreased cardiac output, inhibition of leukocyte migration, and protection of GSH levels by the IBU doses selected may act in concert to enhance the survival of mice. Conversely, the lack of additional dosing with IBU after 5 h may have contributed to the higher death rate seen between 12 and 24 hours. It is clear that further investigations are required to test this hypothesis.

The results from this study represent the third type of phosgene inhalation injury model to be treated successfully with IBU. Earlier studies, one an isolated perfused rabbit lung and the other, pre/post-treatment administration in rats, focused on the amelioration of phosgene-induced pulmonary edema (37,70). This study focused solely on the survival of mice treated with IBU following a lethal dose of phosgene. The data described clearly indicates that IBU administration after a lethal dose of phosgene can enhance the survival of mice. Therapeutic treatment with IBU could supplement current medical management practices that would go into effect following an accidental toxic gas exposure.

Table 3. Mice 12- and 24-hour survival rates following post-treatment of phosgene exposure with ibuprofen. Phosgene exposure was for 20 minutes at 32 mg/m³ (640 mg×min/m³).

IBU conc.	12 hours				24 hours			
	alive/total	survival (%)	(χ ²)	survival odd ratio*	alive/total	survival (%)	(χ ²)	survival odds ratio*
0/0 saline-treated + phosgene	4/16	25	---		1/16	6.2	---	
3/1.5mg/mouse+ phosgene	7/16	44	N.S.	2.3	2/16	12.5	N.S.	2.1
9/4.5 mg/mouse+ phosgene	10/16	63	p<.05	5.0	3/16	19	N.S.	3.5
15/7.5 mg/mouse+ phosgene	13/16	82	p<.005	13	3/16	19	N.S.	3.5

* Odds ratio for survival at each drug dose for 12 or 24 hours was calculated based on the ratio of (total alive/total dead) for each IBU concentration versus exposed and saline-treated. For example, at 15/7.5 mg IBU/mouse, this is (13÷3)/(4÷12)=13. N.S. = Not significant.

Several major conclusions can be drawn from these studies. (A) The site of phosgene-induced lung injury is the epithelial membrane. (B) Inhalation exposure to phosgene can be successfully treated through the intratracheal route. (C) ISO effectively ameliorates phosgene-induced lung edema apparently by acting as a vasodilator, reducing leukotriene production, and by either maintaining or potentiating an adequate lung tissue redox state. (D) ISO prevented lung tissue decreases in cAMP concentration seen with phosgene exposure alone. This single mechanism alone could be partly responsible for the decreased edema measured in the ISO-treated rabbits. (E) These data provide potential information for a rational therapeutic approach in the treatment of toxic gas poisoning. However, caution must be applied when considering the administration of adrenergic compounds such as ISO for treating pulmonary edema. The capacity of ISO (IV) (data not shown) to enhance lung edema, let alone attenuate it, may be linked to its effects on various vascular beds (77). (F) AMIN may protect against oxidant lung injury by a combination of the following: a direct anti-permeability effect, inhibiting the permeability enhancing sulfidopeptide leukotrienes, by direct or indirect antioxidant action, reducing lipid peroxidation and maintaining a cAMP concentration required to keep cellular tight junctions intact, and possibly through vasodilatory mechanisms (62).

Table 4. The effect of phosgene exposure at 12 hours on lung tissue TBARS, NPSH and WW/DW ratios in mice treated 20 minutes after exposure with ibuprofen.

	Treatment Dose of Ibuprofen			
	<u>0/0 (saline)</u>	<u>3/1.5 mg/kg</u>	<u>9/4.5 mg/kg</u>	<u>15/7.5 mg/kg</u>
TBARS nmols/mg prot.	10.5±1.1	10.9±0.4	8.1±0.2*	9.6±0.7
NPSH μmols/mg prot.	1.4±0.5	2.5±0.2*	2.4±0.2*	4.5±0.7**
<u>WW/DW</u>	<u>8.4±.03</u>	<u>8.8±0.2</u>	<u>8.9±0.2</u>	<u>7.3±0.9*</u>

(*) and (**) significantly different from 0/0 saline-treated phosgene-exposed mice, $p \leq 0.05$ and $p \leq 0.01$, respectively. Data were analyzed by one-tailed t-test. Compiled from reference (78).

These observations suggest that AMIN or other drugs that increase cAMP may be useful for the treatment of phosgene or other oxidant gas exposures in man. (G) (IT) NAC was effective in ameliorating LWG when delivered to the site of injury. Mechanisms more than likely revolve around a direct antioxidant effect with the up-regulation of the GSH redox cycle constituents playing an important but minor role. (H) Postexposure treatment with IBU substantially enhanced the survival rate of mice exposed to a LC₅₀₋₆₀ phosgene. IBU's role in antioxidant protection may be one mechanism of its effect, but further work is needed for verification. Additionally, the role of IBU on anti-inflammatory processes in this injury was not assessed but may also be a contributory factor.

References

1. Jackson KE: Phosgene. J Chemical Education 1933; 622-626, October.
2. NIOSH: Criteria for a recommended standard: Occupational exposure to phosgene. USDEW, PHS, CDC publication #76137, 1993, Washington, DC.
3. Singh HB: Phosgene in the ambient air. Nature, 1976; 264: 428-429.
4. Brown JE, Birk MM: Phosgene in the thermal decomposition products of poly (vinylchloride): generation, detection, and measurement. J Anal Toxicol 1980; 4:166-174.
5. Krajewski JA: Chemical accidents and catastrophes as a source of the greatest hazard to the environment and human health. Med Pr 1997; 48(1):93-103.
6. Cucinell SA: Review of the toxicity of long-term phosgene exposure. Arch Environ Health 1974; 28:272-275.
7. Tobias JM: The pathological physiology of the lung after phosgene. In: *Fasciulus On Chemical Warfare Medicine*. Vol. II. Respiratory Tract, p. 331. National Research Council Committee on Treatment of Gas Casualties. 1945, Washington, DC.
8. Diller WF: Medical phosgene problems and their possible solutions. J Occupat Med 1978; 20(3): 189-193.
9. Daly de Burgh I, Eggleton P, Elsden SR, Hebb CO: Biochemical study of isolated perfused lungs with special reference to the effects of phosgene. Quart J Exp Physiol 1946; 33: 215-240.
10. Potts AM, Simon WS, Gerard RW: The mechanism of phosgene and diphosgene. Arch. Biochem 1949; 24:329-337.
11. Babad H, Zeiler AG: The chemistry of phosgene. Chem Rev 1973; 73(1):75-91.
12. Diller WF, Bruch, J, and Dehnen, W. Pulmonary changes in the rat following low phosgene exposure. Arch Toxicol 1985, 57: 184-190.
13. Pawlowski R, Frosolono MP. Effects of phosgene on rat lungs after a single high-level exposure. II. Ultrastructural alterations. Arch Environ Hlth, 1977, 32:278-283.
14. Currie WD, Hatch GE, Frosolono, MF. Changes in lung ATP concentration in the rat after low-level phosgene exposure. J Biochem Toxiol. 1987; 2: 105-114.
15. Frosolono MP, Pawlowski R. Effect of phosgene on rat lungs after single high-level exposure: I. Biochemical alterations. Arch Environ Hlth 1977, 32:271-277.

16. Burleson GR, Keyes LL: Natural killer activity in fischer-344 rat lungs as a method to assess pulmonary immunocompetence: Immunosuppression by phosgene inhalation. *Immunopharmacol Immunotoxicol*. 1989; 11(2&3): 421-443.
17. Selgrade MK, Starnes DM, Illing JW, Daniels MJ, Graham JA; Effects of phosgene exposure on bacterial, viral, and neoplastic lung disease susceptibility in mice. *Inhal Toxicol*, 1989; 1:243-259.
- 18 Diller WF and Borak, J. Phosgene Exposure: Mechanisms of Injury and Treatment Strategies. *J. Occup Environ. Med.* 2000, 43: 110-119.
19. DeCurtis V, Gemma S, Sbraccia M, Testai E, Vittozzi L: The contribution of electrophilic and radadic intermediates to phospholipid adducts formed by halomethanes in vivo. *J. Biochem Toxicol* 1994; 9(6): 305-310.
20. Guastadisegni C, Balduzzi M, Vittozzi L: Preliminary characterization of phospholipid adducts by [14C]-CHCl₃ reactive intermediates in hepatocyte suspensions. *J. Biochem Toxicol* 1996; 11(1): 21-25.
21. Guastadisegni C, Guidoni L, Balduzzi M, Viti V, DiConsiglio E, Vittozzi L: Characterization of a phospholipid adduct formed in Sprague Dawley rats by chloroform metabolism: NMR studies. *J. Biochem. Mol. Toxicol* 1998; 12(2): 93,102.
22. Jugg, B, Jenner, J, and Rice, P. The effect of perfluoroisobutene and phosgene on rat lung fluid surfactant phoslipids. *Human and Exp Toxicol*. 1999, 18:659-668.
23. Polednak AP, Hollis DR: Mortality and cause of death among workers exposed to phosgene in 1943-45. *Toxicol. Indust. Health* 1985; 1(2): 137-151.
24. Guo Y-L, Kennedy TP, Michael JR, Sciuto AM, Ghio A, Atkinson NF, Gurtner GH. Mechanisms of phosgene-induced toxicity: role of arachidonate mediators. *J Appl Physiol* 1990, 69(5):1615-1622.
25. Kennedy TP, Michael JR, Hoidal JR, Hasty D, Sciuto AM, Hopkins C, Lazar R, Bysani GK, Tolley E, Gurtner GH. Dibutyryl cAMP, aminophylline, and β -adrenergic agonist protect against pulmonary edema caused by phosgene. *J Appl Physiol*, 1989, 67(6):2542-2552.
26. Rhinehart RH, Hatch T. Concentration-time product (CT) as an expression of dose in sublethal exposure to phosgene. *Am Ind Hyg Assoc J*, 1964, 25:545-553.
27. Sciuto AM, Strickland PT, Kennedy TP, Guo Y-L, Gurtner GH. Intratracheal administration of dibutyryl cAMP attenuates edema formation in phosgene-induced acute lung injury. *J Appl Physiol*, 1996, 80(1):149-157.

28. Sciuto AM, Strickland PT, Kennedy TP, Gurtner GH. Protective effects of N-acetylcysteine treatment of phosgene exposure in rabbits. *Am J Respir Crit Care Med* 1995; 151(3):768-772.
29. Hayes EC, Lombardo D, Girard Y, Maycock J, Rokach A, Rosenthal R, Young R, Egan W, Swearink H. Measuring leukotrienes of slow reacting substance of anaphylaxis: development of a specific radioimmunoassay. *J Immunol* 1983; 13, 429-433.
30. Peters SE, Schulman E, Liu M, Hayes E, Lichtenstein L. Separation of major prostaglandins, leukotrienes and monoHETEs by high performance liquid chromatography. *J Immunol* 1983, 64:335-343.
31. Tietze P: Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal. Biochem*, 1969, 27:502-522.
32. Akerboom ME, and Sies H: Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods Enzymol*, 1989, 77: 373-382.
33. Buege J, and Aust SD. Microsomal lipid peroxidation. *Methods in Enzymol*, 1978, 51:302-310.
34. Lowry O, Rosebrough NJ, Farr AL, and Randall RJ. Protein measurement with folin phenol reagent. *J Biol Chem*, 1951, 193:265-275.
35. Zar TH. *Biostatistical Analysis*. Second Ed., Englewood Cliffs, NJ, Prentice Hall, 1984.
36. Orzalesi G, Selleri R, Caldini O, Volpato I, Innocenti F, Colome J, Sacristan A, Varez G. Ibuproxam and ibuprofen: A pharmacological comparison. *Arzneim.-Forsch./Drug Res.* 1977; 27(5),1006-1012.
37. Sciuto AM, Stotts RR, and Hurt HH. The efficacy of ibuprofen and pentoxyfylline in the treatment of phosgene-induced acute lung injury. *J Appl Toxicol*. 1996; 16(5), 381-384.
38. Wong SHY, Knight JA, Hopfer SM, Zaharia O, Leach CN, and Sunderman FW. Lipoperoxides in plasma as measured by liquid chromatographic separation of malondialdehyde-thiobarbituric acid adduct . *Clin Chem*. 1982; 33(2), 214-220.
39. Uchiyama M, and Mihara M. Determination of malondialdehyde precursors in tissue by thiobarbituric acid test. *Anal Biochem*. 1978; 86, 271-278.
40. Sedlak J, and Lindsay RH. Estimation of total, protein bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem*. 1968; 25, 192-205.
41. Kahn HA. *Introduction to Epidemiologic Methods*, New York, Oxford University Press; 1983.

42. Sciuto AM, Cascio MB, Moran TS, and Forster JS. The fate of antioxidant enzymes in bronchoalveolar lavage fluid (BALF) over seven days in mice with acute lung injury. Submitted to Inhalation Toxicology, July, 2002.
43. Duniho SM, Martin J, Forster JS, Cascio MB, Moran TS, Carpin LB, and Sciuto AM. Acute changes in lung histopathology and bronchoalveolar lavage parameters in mice exposed to the choking agent phosgene. Toxicologic Path, 2002, 30(3): 339-349.
44. Urbanetti JS. Phosgene: Clinical importance and management. Proceedings of Workshop on Acute Lung Injury and Pulmonary Edema (AD A216293), Aberdeen Proving Ground, MD, May 1989; 105-119.
45. Trethewie ER: Pharmacology of phosgene. The Med. J. Australia. 1947; 20:612-618.
46. Farrukh IS, Sciuto AM, Spannhake WE, Gurtner GH, and Michael JR. LTD₄ increases pulmonary vascular permeability and pressure by different mechanisms. Amer Rev Resp Dis, 1986, 134:229-232.
47. Keuhl FA, Zanetti ME, Soderman DD, Miller DK, and Ham EA. Cyclic AMP-dependent regulation of lipid mediators in white cells. Am Rev Resp Dis, 1987, 136:210-213.
48. Nielson CP, Crowley JJ, Morgan ME, and Vestal RE. Polymorphonuclear leukocyte inhibition by therapeutic concentrations of theophylline is mediated by cyclic-3'-5'-adenosine monophosphate. Am Rev Resp Dis, 1987, 137:25-30.
49. Foy T, Marion J, Brigham K, and Harris TR. Isoproterenol and aminophylline reduce lung capillary filtration during high permeability. J Appl Physiol: Respirat Environ Exercise Physiol, 1979, 46(1):146-151.
50. Mizus I, Summer W, Farrukh I, Michael JR, and Gurtner GH. Isoproterenol or aminophylline attenuate pulmonary edema after acid injury. Am. Rev. Resp. Dis., 131:256-259 (1985).
51. Farrukh, IS, Gurtner, GH, and Michael, JR. Pharmacological modification of pulmonary vascular injury: possible role of cAMP. J Appl Physiol, 1987, 62(1):47-54.
52. Diller WF. Pathogenesis of phosgene poisoning. Toxicology and Industrial Health, 1985, 1:7-15.
53. Singer, AL, and Ariano MA. Localization of cyclic adenosine monophosphate phosphodiesterase in mouse alveolar cells. J Histochem Cytochem. 1981, 29(12): 1372-1376.
54. Erneux C, van Sande J, Mist F, Cochaux P, Decoster C, and Dumont JE. A mechanism in the control of intracellular cAMP levels: the activation of a calmodulin-sensitive phosphodiesterase by a rise of intracellular free calcium. Mol Cell Endocrinol, 1985, 43: 123-134.

55. Werlein RJ, Kirby SD, and Madren-Whalley J. Phosgene-induced calcium changes in pulmonary artery endothelial cells. In: *Advances in Animal Testing for Safety and Efficacy*. Salem H, Katz, S, eds. Taylor & Francis, Washington, DC, in press, 1997.
56. Gillissen A, Jaworska M, Schärling B, van Zwoll D, and Schultze-Werninghaus G. Beta-2-agonists have antioxidant function in vitro. 1. Inhibition of superoxide anion, hydrogen peroxide, hypochlorous acid, and hydroxyl radical. *Respirat*. 1997, 64:16-22.
57. Duffey ME, Hainau B, Ho S, and Bentzel C. Regulation of tight junction epithelial junction permeability by cyclic AMP. *Nature*, 1981, 294:451-453.
58. Shasby D, Shasby SS, Sullivan JM, and Peach MJ. Role of endothelial cell cytoskeleton in control of endothelial permeability. *Circ. Res.*, 1982, 51:657-661.
59. Mueller E and van Breeman C. Role of intracellular Ca++ in Beta-adrenergic relaxation of a smooth muscle. *Nature*, 1979, 281: 682-683.
60. Scheid CR and Fay FS. Beta-adrenergic effects on transmembrane ^{45}Ca fluxes in isolated smooth muscle cells. *Am. J. Physiol.*, 1984, 246:C431-438.
61. Olsson RA, and Pearson JD. Cardiovascular purinoceptors. *Physiol Rev*, 1990, 70:761-845.
62. Webb RC, and Bohr DP: Relaxation of vascular smooth muscle by isoproterenol, dibutyryl cAMP, and theophylline. *J Pharmacol Exp Therapeut*, 1981, 217:26-35.
63. Hitchcock M. Adenosine 3'-5'-cyclic monophosphate phosphodiesterase in guinea pig lung--- properties and effects of adrenergic drugs. *Biochem Pharmacol*, 1969, 22:959-969.
64. Gurtner GH, Sciuto AM, and Knoblauch A. The Role of cAMP in the Regulation of Pulmonary Vascular Permeability. In: *Lung Vascular Injury: Molecular and Cellular Response*, Johnson A, Ferro T, eds, New York, Marcel Dekker, Inc., 1992, Vol. 60, pp 99-111.
65. Sheldon R, Moy A, Lindsey K, Shasby S, and Shasby DM. Role of myosin-light chain phosphorylation in endothelial cell retraction. *Am J Physiol: Lung Cell and Mol Physiol*, 1994, 266:L606-L612.
66. Paky A, Michael JR, Burke-Wolin T, Wolin MS, and Gurtner GH. Endogenous production of superoxide by rabbit lungs. *J Appl Physiol*, 1993, 74:2868-2874.
67. Brown LAS and Longmore WJ. Adrenergic and cholinergic regulation of lung surfactant secretion in the isolated perfused rat lung and alveolar Type II cells in culture. *J. Biol. Chem.* 1981, 256:66-72 .

68. Van Surell C, Boczkowski J, Pasquier C, Du YC, Franzini E, and Aubier M. Effects of N-acetylcysteine on diaphragmatic function and malondialdehyde content in Escherichia coli endotoxemic rats. *Am Rev. Resp. Dis.* 1992; 146:730-734.
69. Moldéus P, Cotgreave IA, and Berggren M: Lung protection by a thiol-containing antioxidant; N-acetylcysteine. *Respiration*, 1986, 50: Suppl 1, 31-42.
70. Kennedy TP, Rao NV, Noah W, Michael JR, Jafri M, Gurtner GH, and Hoidal J. Ibuprofen prevents oxidant lung injury and in vitro lipid peroxidation by chelating iron. *J Clin Invest.* 1990; 86: 1565-1573.
71. Halliwell B, and Gutteridge JMC. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch Biochem Biophys.* 1986; 246(2):501-514.
72. Adams SS, Bough RG, Cliffe EE, Lessel B, and Mills RFN. Absorption, distribution and toxicity of ibuprofen. *Toxicol Appl Pharmacol.* 1969; 15:310-330.
73. Passmore JC and Jimenez AE. Effectiveness of dual cyclooxygenase and leukotriene blockade with ibuprofen and LY203647 during canine endotoxic shock. *Circ Shock.* 1993; 39:21-28.
74. Brown KA and Collins AJ. Action of non-steroidal anti-inflammatory drugs on human and rat peripheral leukocyte migration in vitro. *Ann Rheum Dis.* 1977; 36: 239-243.
75. Ghio AJ, Kennedy TP, Hatch GE, and Tepper JS. Reduction of neutrophil influx diminishes lung injury and mortality following phosgene inhalation. *J Appl Physiol.* 1991; 71(2): 657-665.
76. Lehnert BE, Archuleta D, Gurley LR, Session W, Behr MJ, Lehnert NM, and Stavert DM. Exercise potentiation of lung injury following inhalation of a pneumoedematogenic gas: perfluoroisobutylene. *Exp Lung Res.* 1995; 21:331-350.
77. G. Goldman, R. Welbourn, S. Alexander, et al. Modulation of pulmonary permeability in vivo with agents that affect the cytoskeleton. *Surgery* 1991, 109:533-538.
78. Sciuto, AM. Ibuprofen treatment enhances the survival of mice following exposure to phosgene. *Inhal. Toxicol.* 1997, 9: 389-403.
79. Gilchrist HL. The residual effects of warfare gases: the use of phosgene gas, with reports of cases. *Med Bull of the Vet Admin.* 1933; 10(1), 4-36.